

**CRANFIELD UNIVERSITY**

Patrick Lipphaus

**Flow cytometry for rapid analysis of microbiological water  
quality**

**SCHOOL OF APPLIED SCIENCES**

**MSc by research thesis**

Academic year: 2012-13

Supervisor: Andreas Nocker

March 2014

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## **Introduction**

In aquatic environments, microorganisms are presented with sufficient levels of nutrients to maintain activity and are therefore abundant in water. Organisms present in water may be eukaryotic or prokaryotic and include protozoa, amoebae, bacteria and fungi. The majority of organisms present are of no concern to human health, but some pathogens that usually are introduced by faecal contamination may be present, and have to be removed prior to consumption.

In order to produce safe drinking water, the water industry is challenged with the removal of microorganisms as well as the nutrient and energy sources that could promote microbial regrowth [Allen et al, 2004]. A cascade of treatment processes is applied to purify the water to a level at which it is safe to be consumed. In general the first step of the treatment process is a chemical treatment to remove suspended solids and organic compounds by precipitation. This is followed by a filtration process which is applied to further remove solids and organic compounds. As a final step the disinfectant is added to the water which is applied to remove microorganisms as well as nutrients and chemical pollutants. In order to prevent regrowth in the distribution system a sufficient level of disinfectant has to be maintained throughout the network [WHO, 1997]. Monitoring the treatment process is essential to guarantee the customers safety. Traditionally, plating methods which are widely accepted and anchored in water regulations are used to enumerate total cell numbers (HPC) and to assess hygienic parameters by screening for organisms that indicate faecal contamination. Unfortunately the plating methods have certain disadvantages. As all plating methods are cultivation based they require a considerable period of time until results are delivered. Using the HPC method for the determination of the total cell

number of a sample further takes only about 1% of the bacterial population into consideration, as most bacteria are either not culturable or entered a non-culturable state in response to the low nutrient environment in treated water [van der Kooij, 2003, Wagner et al 1993]. Therefore a more rapid method for the analysis of microbial water quality is needed. In recent years, bacterial flow cytometry (FCM) as a rapid tool has gained increased acceptance as a water quality measure and a number of key studies have underlined its usefulness as an increasingly affordable diagnostic technology [Berney et al, 2008, Hammes et al, 2008, Hoefl et al, 2003].

In this project FCM was applied as potential replacement for HPCs. Two different fluorescent staining methods have been used to enumerate total and intact cell numbers in water samples. In order to determine the total cell concentration (TCC) bacteria in a sample were stained with SYBR Green I (SG), which is a DNA intercalating dye that can penetrate into cells with compromised and intact cell membranes. Thus, it stains the total bacterial population of a water sample. To determine the intact cell concentration (ICC) of a sample, the bacteria were stained with a combination of SG and Propidium Iodide (PI). PI, being also a DNA intercalating dye, can only penetrate into cells with compromised cell membranes, as its structure prevents it from entering intact cells. The SG which as well is present in cells with compromised membranes produces a less intense fluorescent signal which is quenched by the stronger signal produced by the PI. Thus, when stained with the dye combination, compromised and intact cells produce different fluorescent signals which enable the detection and enumeration of the intact cell population.

## **Aims and Objectives**

This project was performed to assess the potential of bacterial flow cytometry as an asset to monitor the microbiological water quality in distribution networks using chlorine as a disinfectant. Furthermore its potential as a replacement for the heterotrophic plate counting method (HPC) was examined. As flow cytometry is a faster, more rapid method to establish the bacterial population of drinking water, it could serve better as an operational tool for the control of water treatment than the traditional plating methods allowing faster responses to changes in the bacterial levels in water. This project also focussed on the differences in bacterial concentrations from the customer's perspective by examining tap water and from the industry's perspective by examining whole scale distribution networks. It was further investigated if flow cytometric data can be correlated to standard plate counts by examining potential relationships between plate counts and total and intact cell concentrations determined by flow cytometry. To accomplish these aims 2 major studies were performed.

First, the water quality was assessed from a consumer's perspective by examining different tap water samples for total and intact cell numbers using flow cytometry and determining the effects of flushing and stagnation. Second, from the industry's perspective, flow cytometric analysis was applied to examine bacterial numbers in distribution systems and to determine regrowth potentials of different waters.



## **1. Microbiological tap water profile of a medium-sized building and effect of water stagnation**

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All data was gathered and arranged by the author of the MSc thesis in correspondence and under supervision of the project supervisor / corresponding author. Some paragraphs of the text body were drafted by the author of the thesis. The majority of the writing was done by the corresponding author.

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*Key words:*

Drinking water

Tap water

Flow cytometry

Stagnation

Bacteria

## 1.1 Abstract

Whereas microbiological quality of drinking water in water distribution systems is routinely monitored for reasons of legal compliance, microbial numbers in tap water are grossly understudied. We applied in this study flow cytometry as a rapid analytical method to quantify microbial concentrations in water sampled at diverse taps in a medium size research building receiving chlorinated water. Taps differed considerably in frequency of usage and were located in different laboratories, bathrooms, and one in a coffee kitchen. Substantial differences were observed between taps with concentrations (per mL) in the range from  $6.29 \times 10^3$  to  $7.74 \times 10^5$  for total cells and from  $1.66 \times 10^3$  to  $4.31 \times 10^5$  for intact cells. The percentage of intact cells varied between 7 and 96%. Water from taps with very infrequent use showed the highest bacterial numbers and highest proportions of intact cells. Stagnation tended to increase microbial numbers in water from those taps which were otherwise frequently used. Microbial numbers in other taps that were rarely opened were not affected by stagnation as their water can probably be considered stagnant most of the time. For cold water taps, microbial numbers and the percentage of intact cells tended to decline with flushing with the greatest decline for taps used least frequently whereas microbial concentrations in water from hot water taps tended to be somewhat more stable. We conclude that microbiological water quality is mainly determined by building-specific parameters. Tap water profiling can provide valuable insight into plumbing system hygiene and maintenance.

## 1.2 Introduction

Whereas testing for faecal indicators like coliforms is the most widely applied strategy to test for potential presence of pathogens, general microbiological quality of drinking water is conventionally assessed using heterotrophic plate counts (HPC; WHO 2002; Bartram *et al.* 2004). The latter dates back to Robert Koch and has been successfully applied for more than 100 years (Koch 1883). HPCs are useful in situations where the focus is not on the hygienic quality of water, but rather on the general microbiological quality and biological stability (Siebel *et al.* 2008). They can serve as a decision basis for operational problems in water treatment processes, for identifying regrowth in distribution systems and for assessment of general cleanliness of pipes (WHO 2002). Nevertheless HPCs suffer from the drawback that test results are obtained only after 2-7 days (depending on the incubation temperature and application) and different results are obtained depending on the nutrient medium (Carter *et al.* 2000; Uhl and Schaule 2004). An attractive alternative to HPCs can be seen in the cultivation-independent quantification of total bacterial cell concentrations by flow cytometry (FCM) which has profited in recent years from the development of user-friendly protocols and instrumentation (for reviews see Hammes and Egli 2010; Wang *et al.* 2010). A number of key studies have underlined the usefulness of this rapid and increasingly affordable diagnostic technology and shown good correlations with total adenosine tri-phosphate concentrations (Berney *et al.* 2008; Vital *et al.* 2012). Compared to traditional HPCs, FCM typically detects, due to its cultivation-independent character, 1-2 log units more microorganisms (Hammes *et al.* 2008).

Although there is an increasing number of studies applying FCM to monitoring cell numbers in drinking water treatments processes (Ho *et al.* 2012; Vital *et al.* 2012), there are very few studies addressing microbiological quality of drinking

water at the level of the consumer's tap. Most notably two Swiss studies employing FCM demonstrated that FCM is a straightforward method to quantify microbial cell numbers in tap water. Siebel *et al.* (2008) reported total cell concentrations (TCC) in the range between  $0.37 \times 10^5 - 5.61 \times 10^5$  cells mL<sup>-1</sup> in tap water samples collected at different time points from two different research buildings at Eawag (Dübendorf, Switzerland). Numbers tended to be highest in the morning and dropped with increasing use. A later study by Lautenschlager *et al.* (2010) showed that microbial numbers in tap water collected from 10 different households tended to increase as a result of stagnation and decreased after flushing taps.

Whereas in both of the two Swiss studies the analysed tap water did not contain disinfectant residual, we applied FCM in this study to quantify numbers of total and intact cells in chlorinated tap water. A research building which accommodates the Cranfield Water Science Institute served as an example to establish a tap water profile. The building was deemed suitable due to availability of a high number of taps that greatly differ in usage. Twenty-two taps were selected to give a representative picture of the building including taps from within laboratories (with some of them being used very infrequently), hand wash sinks located at the entrance/exit of laboratories, two bathrooms, and the coffee kitchen. Although all taps received water from the same mains pipe, only water from the tap located in the coffee kitchen is used for human consumption. Apart from comparing microbial numbers in different locations of the building we were interested in the effect of stagnation, the difference between cold and hot water and in the impact of flushing the taps.

### 1.3 Materials and methods

#### *Sampling*

Water was sampled from kitchen taps in twelve private households in Norfolk, UK. House owners were provided with sterile 100 mL sample bottles (Aurora Scientific, Bristol, UK). Samples were collected on the day of sampling and stored on ice in a cooler until analysis on the following day. As no cleaning procedures were applied, the results reflect the microbiological water quality typically experienced by the corresponding consumers. The same applied to the water sample collected from the coffee kitchen tap at the Cranfield Water Science Institute shown in Fig. 1. All other water samples collected from selected taps in the Cranfield Water Science Institute were collected after disinfecting metal parts of taps prior to sampling with household surface wipes (Kirkland signature brand; Costco, Milton Keynes, UK) containing quaternary ammonium compounds (0.14% dimethyl ethylbenzyl ammonium chlorides, 0.14% dimethyl benzyl ammonium chlorides) and 8% isopropyl alcohol followed by rinsing with 70% ethanol. After a brief additional wipe with clean tissue, the ethanol was allowed to evaporate for 5 min. To avoid any ethanol residues in the sampled water, the first 50 or 200 mL (as indicated) of flow through were discarded. Taps were located in different laboratories (abbreviated lab 1-7), two bathrooms and the coffee kitchen. From every tap 40 mL of water was sampled in a sterile 50 mL conical centrifuge tube (Fisherbrand, Fisher Scientific UK Ltd., Loughborough). To each sample 40 $\mu$ L thiosulfate (0.1 N; VWR, East Grinstead, UK) were added to eliminate residual chlorine. When flushing taps with up to 40 L, defined water volumes were discarded during the sampling process before the next sample was collected.

### ***Bacterial staining and flow cytometric measurements***

For measurements of total cell concentrations, SYBR Green I (10,000 x stock, cat. nr. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) to a working stock concentration of 100 x, of which 5  $\mu$ l were added to sample volumes of 500  $\mu$ l (final SYBR Green I concentration: 1x). For measurements of intact cell concentrations, dye mixtures were made containing 5 parts of 100 x SYBR Green I and 1 part of propidium iodide (1 mg mL<sup>-1</sup>, corresponding to 1.5 mM; cat. nr. P3566; Life Technologies Ltd., Paisley, UK). 6  $\mu$ l of this dye mix were added to each water sample of 500  $\mu$ l (final concentrations of SYBR Green I: 1x, final concentration of PI: 3  $\mu$ M). Flow cytometric measurement in combination with staining with PI has been shown previously to be an appropriate method to exclude cells with membrane damage inflicted by chlorination (Ramseier *et al.* 2011). Following the addition of dyes and brief gentle vortexing, samples were incubated for 15 min at 30°C using a standard laboratory heat block with a self-made styropor lid to maintain a constant temperature and to avoid light exposure. Following staining, samples (50  $\mu$ l) were analysed using a BD Accuri C6<sup>®</sup> flow cytometer equipped with a 488 nm solid-state laser (Becton Dickinson U.K. Ltd., Oxford, UK). In cases where total signals exceeded 10<sup>6</sup> counts mL<sup>-1</sup>, samples were diluted with filter sterilized (0.22  $\mu$ m) Evian mineral water (Evian, Évian-les-Bains, France). All samples were measured at least in duplicate. Green fluorescence was collected in the FL1 channel at 533 nm (FL1) and red fluorescence in the FL3 channel at 670 nm (FL'') with the trigger set on the green fluorescence. No compensation was used. The single, fixed gate described previously by Gatza *et al.* (2013) was employed as a template together with the corresponding instrument settings. Numbers of

microorganisms contained in this gate after staining with SYBR Green I or SYBR Green I/propidium iodide are reported. Data was processed using the Accuri C6 software.

### ***Chlorine and temperature measurements***

Water temperatures were measured using a digital infrared IR thermometer (Precision Gold N85FR; Maplin, Rotherham, South Yorkshire, UK). Total and free chlorine concentrations were measured using a Delagua comparator (Delagua, Marlborough, UK) following the manufacturer's instructions.

## 1.4 Results

### *Heterogeneity of bacterial concentrations in water from kitchen taps*

Water samples taken from kitchen taps located in different private households in Norfolk and in the coffee kitchen of the Cranfield Water Science Institute and were tested for microbial concentrations. Figure 1 shows representative flow cytometric density plots of five selected samples with microbiological water qualities typically experienced by the corresponding consumers.

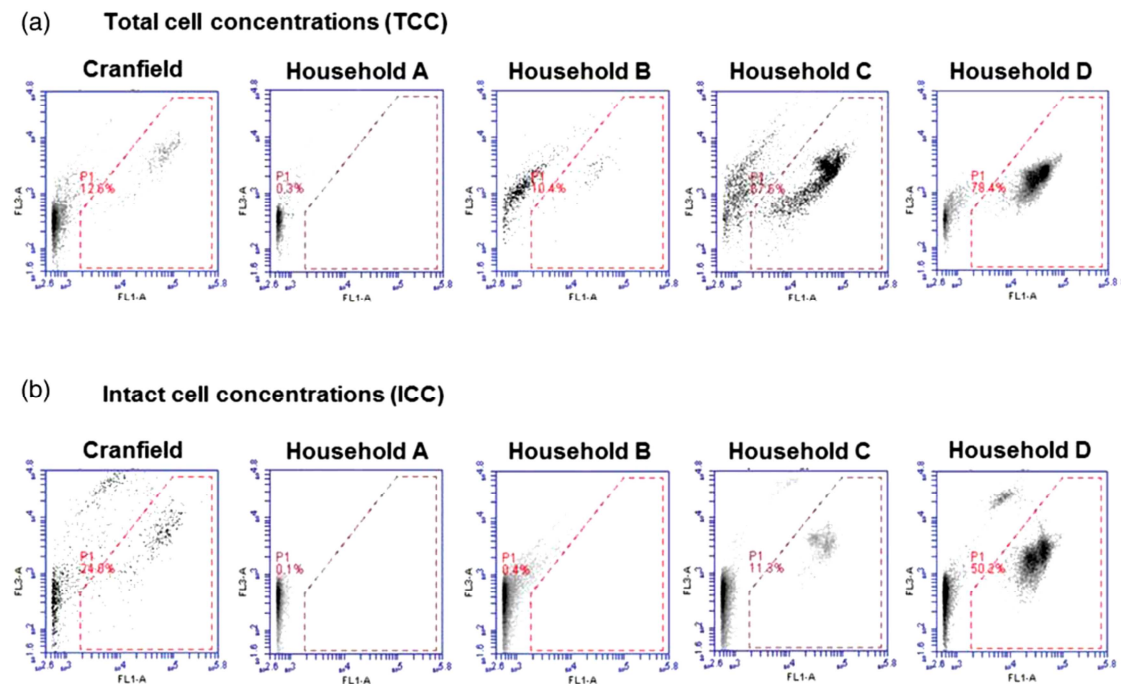


Figure 1. Flow cytometry dot plots of water sampled from kitchen taps in Cranfield University and four different households in Norfolk. FL1 denotes green fluorescence signals (520nm) and FL3 denotes red fluorescence signals (615 nm). An electronic gate (confined by dotted red lines) was used to separate (a) total bacterial cells and (b) intact bacterial cells from background.



Both total cell counts (TCC) and intact cell counts (ICC) were measured with microbial signals confined in the gated regions. Examples were selected as they demonstrate substantial differences in microbial numbers ranging from a few signals in the gate on the low end to  $1.98 \times 10^5$  per mL and  $1.63 \times 10^5$  mL on the high end for total and intact cells, respectively. Interestingly, households C and D which showed dramatically higher bacterial numbers than other samples used point-of-use water filters which had not been changed within the recommended time frame and probably enriched the water with bacteria that built up in the filter cartridge. Especially in household D the majority of microorganisms in this household were found to be intact.

#### ***Tap water fingerprint of a research building***

The pronounced differences in microbial concentrations found in tap water from different households made us ask about the heterogeneity in tap water from a single building receiving chlorinated water from one water pipe. In contrast to the samples shown in Fig. 1, taps were disinfected before sampling to exclude the possibility that microbial contamination derives from tap contamination rather than the water. Maximal concentrations of  $0.5 \text{ mg L}^{-1}$  total chlorine and  $0.1 \text{ mg L}^{-1}$  of free chlorine were measured in water from frequently used taps, whereas the residual concentration was below the detection threshold of  $0.1 \text{ mg L}^{-1}$  for other taps.

The Cranfield Water Science Institute which has a number of different laboratories was deemed an appropriate example not only due to the availability of a relatively high number of taps in different facilities, but also from the perspective that water usage from different taps greatly varies. The tap located in the coffee kitchen can be assumed to be the most frequently used one. Total and intact cell concentrations are

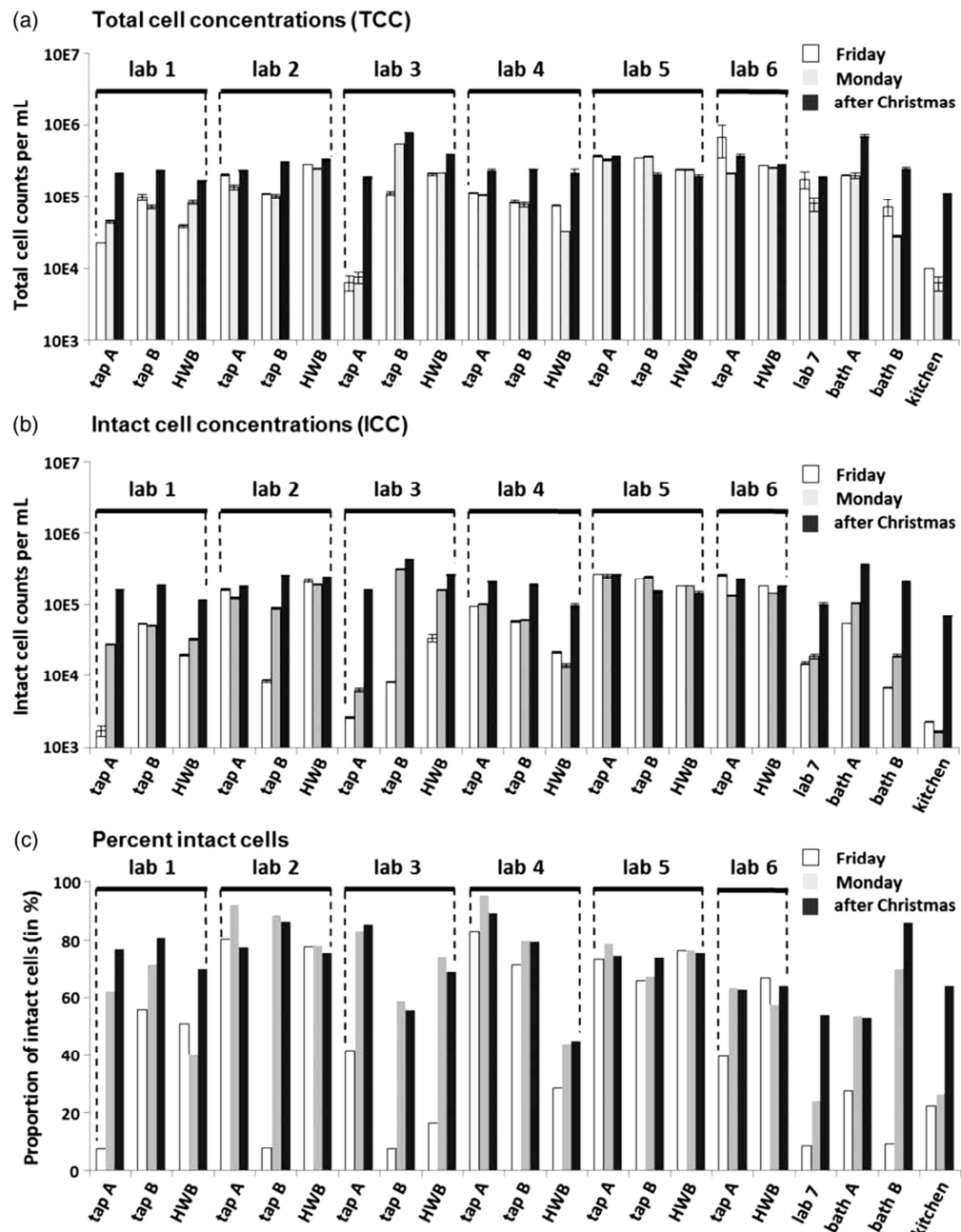


Figure 2: Concentrations of (a) total bacterial cells and (b) intact bacterial cells per mL and corresponding relative proportions of intact cells (c) in water sampled from selected taps in the Cranfield Water Science institute on a Friday and Monday before Christmas and the first day after the Christmas holiday. Samples were collected from taps from different laboratories including hand wash basins (HWB), two different bathrooms and the coffee kitchen. Averages from two technical replicates are shown with error bars representing standard deviations.

shown in Fig. 2a and b, respectively. In order to assess the impact of water stagnation, samples were taken on a Friday and Monday in December 2012 before the Christmas holiday and on the first day after the building was re-opened after an approx. 2 week break over Christmas and New Year. Cell concentrations were found to vary considerably between different taps with differences of up to approx. 100-fold for both total ( $6.29 \times 10^3$  to  $7.74 \times 10^5$  per mL) and intact cells ( $1.66 \times 10^3$  to  $4.31 \times 10^5$  per mL). The effect of stagnation depended on the taps being sampled. Whereas microbial concentrations of water from some taps underwent strong increases after the weekend break and especially after the Christmas holidays, highly comparable concentrations were obtained from others. Stagnation tended to have the strongest impact for frequently used taps located in the kitchen, bathroom B, and in part in laboratories 1 to 4. Microbial concentrations of water from laboratories 5 and 6 on the other hand remained practically unchanged correlating with very rare use of these locations. Water in these laboratories can be considered stagnant for long periods. The differences between total and intact cell concentrations represent the relative proportion of intact cells that for reasons of better visualization are shown in Fig. 2C. Percentages of intact cells hugely varied and were found to range between 7 and 96%.

### ***Effect of flushing taps with increasing water volumes***

To assess the effect of flushing on microbial concentrations, we analysed water from three selected cold water taps (Fig. 3a, b, and c) and an eye wash (Fig. 3d). Water was sampled after flushing taps with water volumes up to 40 L (10 L in case of the eye wash). For both water from laboratory 3 (tap B) and from a storage room microbial numbers declined by factors of 33 and 36 for total cells and by factors of 61 and 429 for intact cells, respectively. In case of the storage room, the very high total cell

concentration of  $1.9 \times 10^6$  in the initial sample (taken after flushing with 0.2 L) can be explained by the fact that the corresponding tap had not been used for at least several months.

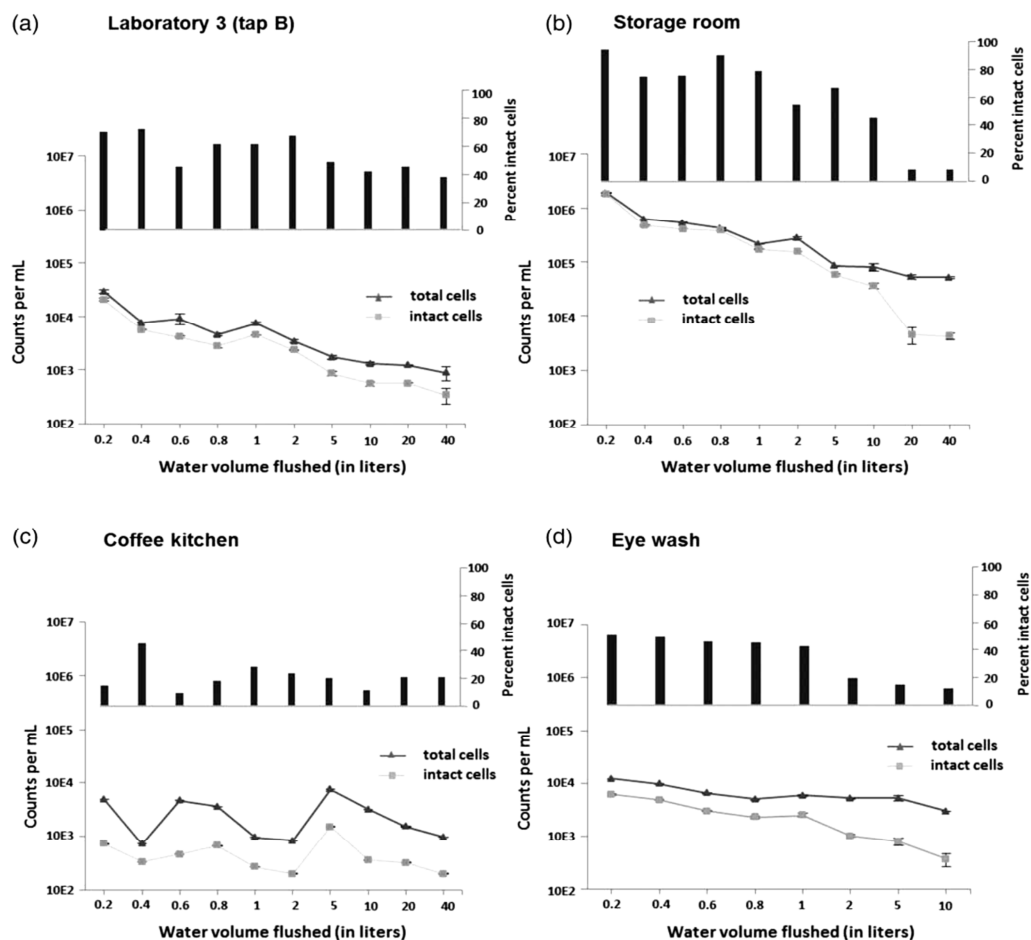


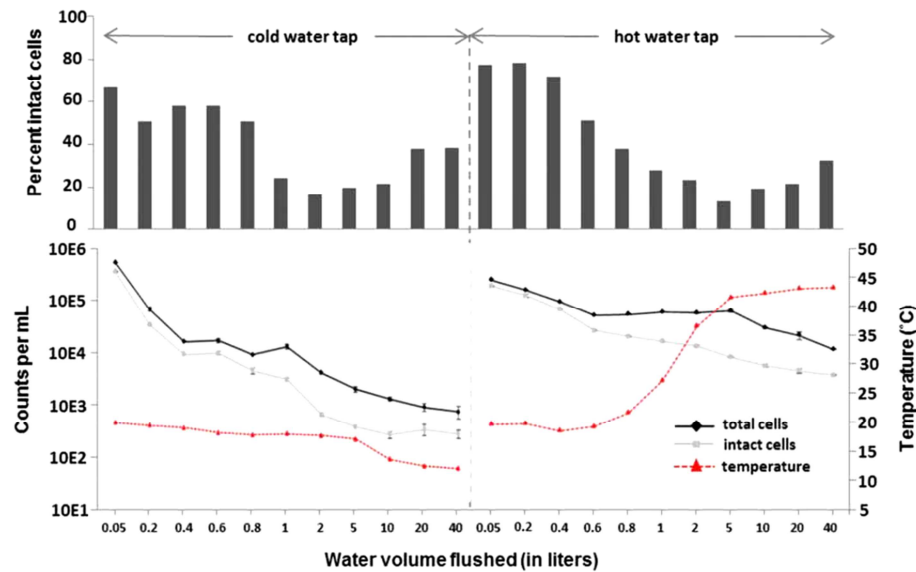
Figure 3: Concentrations of total and intact bacteria per mL detected in water from cold water taps in (a) laboratory 3, (b) a storage room, (c) the coffee kitchen and (d) an eyewash after flushing taps with water volumes ranging from 0.2 L to 40 L. Equidistant presentation of data points was chosen to enable good graphical resolution. All taps are located in the same research building. Error bars represent standard deviations from two technical replicates. Average relative differences between total cell counts and intact cells counts are visualized in a separate bar chart showing the percentage of intact cells.

The relative proportion of intact cells tended to decrease with increasing water volumes. The effect was especially pronounced for the storage room with the proportion of intact cells dropping from 95 to 8 %. Water from the coffee kitchen, which is frequently used by members of the institute, on the other hand, showed the lowest initial number of total and intact microorganisms (Fig. 3c). Correlating with its frequent use of the tap, neither the cell concentrations nor the relative proportion of intact cells underwent a pronounced decrease, but fluctuated around a median value. Water from an eye wash, which is checked weekly due to Health and Safety regulations, showed relatively low microbial numbers, which dropped moderately when flushed with up to 10 L of water (Fig. 3d). The relative proportion of intact cells dropped from an initial 50 % to 12%.

### ***Comparison of cold and hot water***

Water from cold and hot water taps belonging to sinks in laboratory 3 (tap B) and the hand wash basin of laboratory 6 were compared for their microbial concentrations (Fig. 4). For cold water, concentrations dropped with increasing water volumes flushed through the taps. The effect was particularly pronounced for cold water from the hand wash basin where microbial numbers dropped to levels near the detection threshold. In both laboratories the water became substantially colder after flushing 10 and more liters of water indicating that water from the mains pipe was entering the building. In case of laboratory 6, the drop in temperature correlated with a sharp drop in the proportion of intact cells (Fig. 4b). For laboratory 3 on the other hand the percentage of intact cells reached a minimum after flushing 1 L of water and subsequently moderately increased.

(a) Laboratory 3 (tap B)



(b) Hand wash basin (laboratory 6)

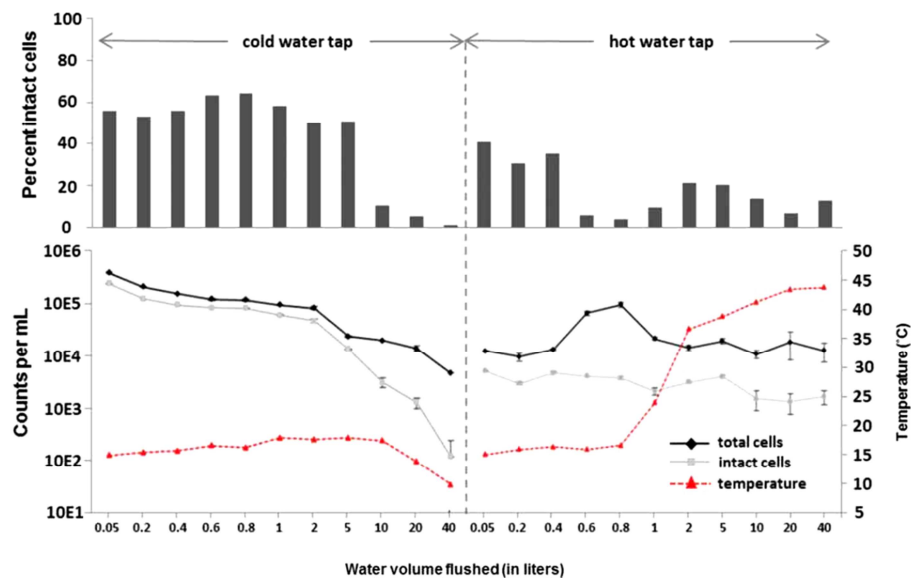


Figure 4: Concentrations of total and intact bacteria detected in water from cold and hot water taps in (a) laboratory 3 and (b) a hand wash basin belonging to laboratory 6. Both sinks are located in the same research building. Samples were taken after consecutive flushing of cold and hot water taps with water volumes ranging from 0.05 L to 40 L. Equidistant presentation of data points was chosen to enable good graphical resolution. Error bars represent standard deviations from two technical replicates. Average relative differences between total cell counts and intact cells counts are visualized in a separate bar chart showing the percentage of intact cells.

Water from the corresponding hot water taps showed more distinct behaviours. Whereas initial microbial concentrations in laboratory 3 were comparable with the ones from the cold water tap and underwent a decline with increasing flushing, concentrations in hot water from the hand wash basin in laboratory 6 were lower and remained relatively constant with increasing water volumes. The difference between the two hot water taps also reflected in the proportions of intact cells which sharply dropped for laboratory 3, but did not show a clear tendency in case of laboratory 6. For both sampling locations the hot water reached a final temperature of below 45°C.

## 1.5 Discussion

As microbiological water quality at the household level is tested only very sporadically for a number of reasons, limited data is available about microbial numbers in household plumbing systems. We addressed in this study the differences in microbial concentrations in water from selected taps using flow cytometry as a modern diagnostic tool which detects microorganisms irrespective of their culturability. Substantial differences in microbial water quality were found in different private households and at different locations of the selected research building. Whereas differences in microbial numbers from taps in private households can be caused by different levels of hygiene (samples were taken without disinfection of taps), point of use devices (such as filters), and varying water quality in different parts of the distribution system, differences in the research building (with all taps being cleaned following the same protocol) suggest that tap water quality is to a large extent determined by building-specific parameters. This finding is in agreement with a study looking at HPC bacteria in different houses in Tucson, Arizona (Pepper *et al.* 2004). Bacterial numbers were found to dramatically increase from the distribution system to the consumer's tap. The authors concluded that the "major source of bacteria ingested by the average consumer in Tucson originates from bacteria within the household distribution system or the household tap, rather than from the source water or the distribution system". HPC levels were reported to greatly vary between households (bacterial concentrations in water from kitchen taps were in the range of  $4 - 7 \times 10^7$  CFU mL<sup>-1</sup> with an average of 300), between different taps within the same household and when sampling the same tap over seven consecutive days.

Differences in microbial numbers between taps varied in our study up to 100-fold (not considering water from the storage room where total and intact cell number of up to



$2.8 \times 10^7$  and  $2.6 \times 10^7$  were measured). Relative differences in microbial numbers appear greater than the ones observed in the Swiss FCM-based studies mentioned earlier.

Total cell concentrations in 200 tap water samples collected at different time points by Siebel *et al.* (2008) were in the range between  $0.37 \times 10^5 - 5.61 \times 10^5$  cells mL<sup>-1</sup> (difference of a factor of 15) and intact cell numbers in tap water from 10 different households analysed by Lautenschlager *et al.* (2010) were in the range of approx.  $0.4 \times 10^5 - 1.6 \times 10^5$  cells mL<sup>-1</sup>. It is tempting to hypothesize that the greater relative differences in our and the Tucson study were influenced by the fact that water was chlorinated, whereas both the Swiss studies were performed with tap water that did not contain disinfection residual. Depletion of disinfectant residual might be an important reason for variation in microbial numbers in buildings.

Non-chlorinated water when entering buildings might on the other hand be biologically more stable and undergo less variation. When studying intact cell concentrations as a function of water age in distribution systems, biological stability was interestingly found to be much higher in the absence of residual when comparing systems with chlorinated water (Riga) or non-chlorinated water (Amsterdam, Zürich; Vital *et al.* 2010). Intact cell numbers in Amsterdam were nearly identical for the entire analysis range of over 85 (Amsterdam) and 50 hours (Zürich) of water age. Also an earlier study by Servais *et al.* (2004) confirmed a strong impact of chlorine. When quantifying the biofilm accumulating on coupons in the Parisian water distribution network, an increase in biomass was reported with decreasing chlorine residual. It is well conceivable that water which is biologically stable in the distribution system is less susceptible to change also in plumbing system.

Stagnation in our study was found to dramatically impact water especially from those taps which were frequently used causing differences of up to 2 log units when comparing intact cell concentrations before and after the Christmas holiday. The same held true to a lesser extent for total cell concentrations. Flushing taps on the other hand caused in part great reductions in microbial numbers with the most dramatic drop from  $2.2 \times 10^5$  cells to the lower detection limit as observed for intact cells in water from the hand wash basin in laboratory 6 after flushing the system with 40 L (Fig. 4b). The effect of stagnation and flushing is again in agreement with both Swiss studies. Siebel *et al.* (2008) reported that total cell concentrations tended to be highest in the morning (8:00 am; approx.  $1.5 \times 10^5$  cells mL<sup>-1</sup>) and typically decreased until 10:00 am ( $0.5 \times 10^5$  cells mL<sup>-1</sup>) before stabilizing during the day. Lautenschlager *et al.* (2010) on the other hand found that intact cell numbers increased between 1.6 and 3.2 times after overnight stagnation with an average of  $1.1 (\pm 0.25) \times 10^5$  intact cells mL<sup>-1</sup> (compared to a 2-18-fold increase in ATP concentrations and a 4-580-fold increase in HPC numbers). Numbers originated from 1 L samples. Flushing taps for 5 min (approx. 30 L) on the other hand decreased cell numbers by a factor of 2-3 while the relative proportion of intact cells remained the same with approx. 85%. The impact of stagnation and flushing was therefore relatively small given the fact that in relatively small households the pipe systems are much shorter than in a research building and water from the mains pipe should enter the building faster. The less pronounced impact of stagnation and flushing in the Swiss studies might again correlate with the absence of disinfectant residual and greater biological stability. Interestingly, supplementary material provided by Lautenschlager *et al.* (2010) showed that flushing had a more pronounced effect on tap water from households in Basel receiving water with a low concentration of chlorine dioxide (0.05 mg L<sup>-1</sup>).

Whereas the first liter of water that was sampled contained on average a relative proportion of 57% intact cells, flushing the taps for 5 min resulted in a drop in intact cells to 34%. However, presence of a disinfectant residual should probably not be overestimated. A very recent report about water quality from taps in a newly constructed building in Winterthur (Switzerland) receiving non-chlorinated water showed that 15 hours of stagnation could also result in increases by a factor of 10-100 in total cell counts as determined by FCM (Kötzsch and Egli 2013). It has to be noted however that the building suffered from serious carbon leaching due to the newly installed plumbing material.

Reasons for differences in microbial numbers can be manifold and be of chemical, physical or operational nature. As mainly shown for distribution systems they can include differences in nutrient and disinfectant concentrations, temperature, and residence times (Kerneys *et al.* 1995; LeChevallier 2003; Niquette *et al.* 2000). In buildings, differences in plumbing materials and nutrients originating from pipe material (Bucheli-Witschel *et al.* 2012) and the household environment can add to this variation (Lautenschlager *et al.* 2010; Rogers *et al.* 1994). Stagnation might however be one of the most important factors influencing microbial numbers as graphically visualized in Fig. 5. In partial reiteration of the previous, reasons for higher microbial numbers in stagnant parts of the plumbing system might include (1) depletion of disinfectant residual due to long residence time, (2) buildup of microorganisms in areas undisturbed by flow, (3) higher water temperatures in buildings compared to the mains pipe (as demonstrated in Fig. 4) and (4) enrichment of stagnant parts with nutrients supporting growth. In regard to nutrient enrichment, the underlying thought is that taps with stagnant water are not a closed system, but amenable to influx of nutrients by passive diffusion. Microorganisms colonizing distal parts with stagnant

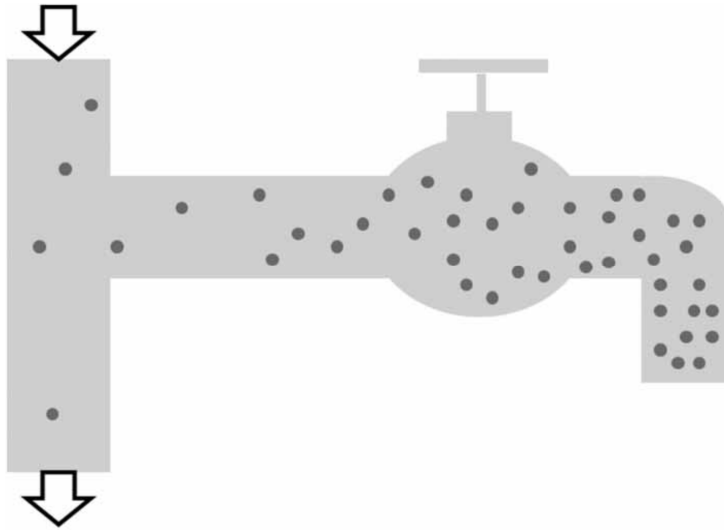


Figure 5. Simplified hypothetical model of microbial concentrations in plumbing system with flowing water and stagnant tap (diagram not drawn to scale). Numbers of microorganisms (represented by dots) are elevated in stagnant parts due to (1) depletion of disinfectant residual, (2) undisturbed buildup of microbial populations due to absence of flow, (3) higher water temperatures compared to mains pipe, and (4) enrichment with nutrients in stagnant parts. Nutrients are able to passively diffuse into stagnant parts and become ‘trapped’ by microorganisms colonizing the plumbing material. Conversion of nutrients into biomass and the resulting ‘fixation’ lowers the probability of nutrients diffusing back into the pipe resulting in a net-influx of nutrients. The build-up of nutrients over time would support a higher microbial concentration. Dead biomass would be recycled rapidly as reflected in a high proportion of live cells.

water would be assumed to convert these nutrients into biomass. The biomass in turn can be expected to be partly attached to the surface of the plumbing material (Moritz *et al.* 2010). Nutrients being converted into biomass could therefore be considered to undergo a ‘microbial fixation’ in stagnant parts. The therefore lower probability to diffuse back into the pipe region with flowing water would result in a net-inflow of

nutrients into distal parts and nutrient enrichment. Higher microbial concentrations when opening taps with stagnant water in turn would be explained by the high likelihood that cells in surface-associated biofilms are in equilibrium with free-floating cells. Whereas this model is hypothetical, it is clear that depletion of disinfectant residual alone cannot explain the microbial buildup, as it is also observed (although to a lesser extent) in households receiving water without secondary disinfectant. Nutrients released directly from plumbing material on the other hand should be considered insufficient to cause the repeated buildup of high microbial numbers, especially when the plumbing has been installed decades ago. Another factor facilitating microbial growth could lie in the size of the colonized surface area: in the beforementioned study by Kötzsch and Egli (2013), the increase in total cells after 15 hours of stagnation was particularly pronounced at the first sampling point when water directly entered the building although plumbing material in this part of the building consisted of stainless chromium steel which should not release organic nutrients. It was discussed that the increase in microbial numbers were alleviated by the big surface of a particle filter directly preceding the sampling point.

In conclusion, the presented data supports previous findings that microbial concentrations in water with disinfectant residual are primarily determined by plumbing systems at the point of use and by building-specific parameters. Stagnation was found to cause dramatic differences both in cell numbers and the relative proportions of intact cells. The study furthermore underlines that FCM represents a sensitive method to detect differences in microbial numbers over an extended concentration scale. Its user-friendliness and speed of data collection adds great value to collect informative large datasets such as required for tap water profiles of buildings.

## **1.6 Acknowledgements**

This study was part of an MSc by Research project funded by Scottish Water which is gratefully acknowledged.

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## **2. Strengths and limitations of flow cytometry for microbiological water quality analysis – a case study**

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*Key words:*

Drinking water

Flow cytometry

Bacteria

## **2.1 Abstract**

In this study bacterial flow cytometry was used as a rapid operational tool to monitor microbiological cell concentrations of 3 different drinking water treatment plants and their adjacent service reservoirs. Bacterial cell concentrations were found to vary considerably between the 3 examined distribution systems. Further the potential of flow cytometry as a replacement for HPCs was investigated by comparing flow cytometric data to traditional plate counts of various raw waters and samples which experienced bacteriological failures. Data from both standard plate counts and flow cytometry further suggested that samples exhibiting coliform failures showed high percentages of intact cells. By comparing coliform counts and flow cytometry data we could determine that a minimum level of 0.5 mg/L of free chlorine in chlorinated distribution systems and 0.8 mg/L total chlorine in chloraminated networks should be maintained to prevent elevated cell concentrations and viabilities. Further it was established that potential regrowth of bacterial cells does not depend on the water source rather than the efficacy of the treatment. The determination of the regrowth potential of different treated waters revealed a considerable bacterial matter regardless of the waters origin.

## **2.2 Introduction**

As in aquatic environments bacteria are presented with a sufficient level of nutrients in order to support microbial activity, they are abundant in water. Drinking water treatment aims to remove bacteria as well as nutrients from water by a cascade of processes and the application of a disinfectant. In order to prevent microbial regrowth in the distribution system a sufficient disinfectant level has to be maintained throughout the network [WHO, 2002].

Traditionally, regulations for monitoring the microbiological water quality and to monitor operational procedures are based on the enumeration of faecal indicator organisms and HPCs [Bartram et al., 2002; Robertson and Brooks, 2003]. These typically are based on plate counting [WHO, 2002, Bartram et al., 2002], which demand a considerable amount of time to obtain results (HPC counts take 2-3 days). Whereas this has worked well from a hygienic perspective, because of the time delay, the approach is of little use for operational decisions. Further, traditional plating methods obtain very low microbial numbers from distribution networks as they only take culturable organisms into account. However, it has been shown that bacteria can enter a viable but non-culturable state in a low nutrient environment and in presence of low disinfectant levels [Byrd et al, 1991]. Therefore a more rapid method is required to serve as a decision basis for operational problems in water treatment processes, to monitor microbial activity, to identify regrowth in distribution systems and to assess the general cleanliness of pipes.

In recent years, bacterial flow cytometry as a rapid tool has gained increased acceptance as a water quality measure and a number of key studies have underlined its usefulness as an increasingly affordable diagnostic technology [Berney et al, 2008; Vital et al, 2012]. This study has looked into the usefulness of FCM as a replacement

of HPC counts for operational purposes as it delivers results within an hour and can therefore enable a real time water quality assessment. Flow cytometry in this project was applied to monitor total and intact cell populations in 3 selected networks within the Scottish Water distribution system over a 7 month time period. By this, the effectiveness of the water treatment was examined, possible seasonal variation in microbial numbers in the distribution system could be recognised and potential errors in the treatment process could be determined. Furthermore, the increase of cell numbers within a network could be used to identify areas of localised bacterial regrowth in the system. Microbial growth in a distribution network is promoted by a variety of factors such as the availability of nutrients or carbon sources pipe material and surface area-to-volume ratios, stagnation and temperature [Allen et al., 2004, Carter et al., 2000, LeChevallier, 2003] As bacterial multiplication in treated water constitutes a risk to the hygienic safety of consumers, flow cytometric analysis was used to determine the regrowth potential of selected treated waters and different raw waters. The collected data was collated with standard plate counts of these waters to examine any correlation of the counts.

It could be assumed that bacterial growth within the distribution network could lead to an increased incidence of bacteriological failures in the drinking water supply chain. A water sample by regulations is considered a failure if it shows one or more colony forming units of coliform bacteria or *E. coli* in 100 mL (cfu / 100 mL) sample volume. FCM analysis was applied to establish potential correlations with standard plate count methods.

## 2.3 Materials and Methods

### *Sampling*

Water samples were taken from a variety of different Treatment Works and Service Reservoir Outlets (for treated water) and Inlets (for raw water) in the Scottish Water distribution network according to Scottish Water sampling procedures. Taps were flushed for 3 minutes and then flame sterilised using a blow torch for 30 seconds. After flushing the tap for an additional 30 seconds, water was sampled into sterile 500 mL plastic-sample bottles (Aurora Scientific, Bristol, U.K.) containing thiosulfate to eliminate chlorine residues. Samples were stored and transported in a refrigerated van at 5°C (+/- 3°C) and arrived in the laboratory within 24 hours after sampling.

### *Chlorine measurements*

The measurement of free and total chlorine residuals was performed at the time of sampling, using a pocket colorimeter (Hach-Lange, Salford, U.K.) set to the Low (LO) range mode. Appropriately labelled sample cells were filled with 10 mL of water and any liquid spills were wiped off the outside of the cells using paper tissue. After reading the blank, the DPD free and total chlorine DPD powders were added to the sample cells and cells were gently swirled for 30 seconds, followed by a 1 min incubation prior to measurement and incubated for 1 minute before analysis.

### *Flow cytometric measurements*

For measurements of total cell concentrations (TCC), SYBR Green I (10,000 × stock, cat. no. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with dimethyl sulphoxide (Fisher Scientific, Fair Lawn, NJ) to a working stock concentration of 100×, of which 5 µL were added to sample volumes of 500 µL (final SYBR Green I concentration: 1×). For measurements of intact cell concentrations (ICCs), dye

mixtures were made containing five parts of  $100 \times$  SYBR Green I and one part of propidium iodide (PI) ( $1 \text{ mg mL}^{-1}$ , corresponding to  $1.5 \text{ mM}$ ; cat. nr. P3566; Life Technologies Ltd., Paisley, UK). Six microlitres of this dye mix were added to each water sample of  $500 \text{ }\mu\text{L}$  (final concentrations of SYBR Green I:  $1\times$ , final concentration of PI:  $3 \text{ }\mu\text{M}$ ). Combining flow cytometric analysis with staining with PI has previously been shown as an appropriate method to exclude cells with chlorine induced membrane damage [Ramseier et al, 2011]. After the addition of the dyes the samples were gently vortexed and incubated on in a standard laboratory heat block for 15 min at  $30^\circ\text{C}$  covered with a custom self-made aluminium foil-wrapped polystyrene lid to ensure homogeneous temperature conditions and to avoid light exposure. Following incubation, samples ( $50 \text{ }\mu\text{L}$ ) were analysed using a BD Accuri C6 flow cytometer equipped with a 488 nm solid state laser (Becton Dickinson U.K. Ltd., Oxford, U.K.). In cases where the total signals exceeded  $10^6$  counts  $\text{mL}^{-1}$ , samples were diluted using filter sterilized ( $0.1 \text{ }\mu\text{m}$ , Millex, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co, Cork, IRL.) Evian mineral water (Evian, Evian-les-Bains, France) as this mineral water tends to be biologically stable with little variation between bottles. Green fluorescence was collected in the FL1 channel at 533 nm (FL1) and red fluorescence in the FL3 channel at 670 nm (FL3) with the trigger set on the green fluorescence. No compensation was used. The single, fixed gate described previously by Gatza et al. was employed as a template together with the corresponding instrument settings (Gatza et al., 2013). Numbers of microorganisms contained in this gate after staining with SYBR Green I or SYBR Green I/PI are counted as TCC or ICC. Data was processed using the Accuri C6 software.



### ***Regrowth potential assessment***

Water was sampled according to Scottish Water procedures into sterile 500 mL plastic-sample bottles containing thiosulfate (Aurora Scientific, Bristol, U.K.) and stored under refrigerated conditions until regrowth potential was assessed. Samples were incubated at 22°C without shaking. Bottles were opened daily to allow air exchange. TCC and ICC measurements were performed as described above at time 0 (within 24 hours after samples reached the laboratory) and after 7 days or after indicated time points (daily up to 10 days).

Our procedure was based on the use of readily available standard industry plastic sampling bottles although we acknowledge that nutrients may be released from the plastic material. Despite the absence of acid-washed, heat-furnished glass bottles free of any assimilable organic carbon, the results were promising and potential errors would have affected all samples in the same way.

### ***Standard microbiological analysis***

Total viable counts (TVCs) of drinking water samples were determined by mixing 1 mL of sample into 18 mL of YEA (Yeast Extract Agar, cat. No. CM0019B, Oxoid Ltd., Thermo fisher Scientific, Loughborough, U.K.). Plates were incubated at 37°C for 48h and at 22°C for 72h. Membrane filtration was used to determine the presence of coliforms and *E. coli* in drinking water samples and raw waters by passing 100 mL of sample through a 0.45 µm filter-membrane (Pall Corporation, Ann Arbor, Michigan, USA) which was placed onto a MLGA plate (Membrane Lactose Glucuronide Agar, cat. No. CM1013B, Oxoid Ltd., Thermo fisher Scientific, Loughborough, U.K.). Plates were incubated at 30°C for 4h and 37°C C for 14h in a MIR-253 cyclic incubator (Sanyo-Biomedical, Loughborough, U.K.).

### ***Chemical analysis***

Determination of the pH of water samples was done determined using a ROSS Ultra pH electrode (Thermo Fisher Scientific Ltd., Loughborough, U.K.). The TOC content was analysed using a Formacs high temperature catalytic combustion system (Skalar Analytical B.V., Breda, Netherlands). Chloride, ammonia, nitrite, SRP and TON were determined by colourimetric analysis using a Aquakem 600 discrete analyser (Thermo Fisher Scientific Ltd., Loughborough, U.K).

## 2.4 Results

### *Comparison of bacterial viability and chlorine residuals*

Over a period of 7 months water samples were taken from 3 different distribution systems in the Scottish Water network. By examining the total and intact cell population in each sample the viability of bacterial cells was determined. In figure 1 the percentages of intact cells are shown together with the corresponding chlorine residuals. As distribution system 1 utilises chlorine as a disinfectant the free chlorine residual is shown. It becomes clear that as long as a free chlorine level of above 0.5 mg/L in system 1 is maintained the percentages of intact cells stay below 15%, whereas a drop beneath 0.5 mg/L results in higher viabilities of up to 100%. The water samples that were taken directly after the treatment process (FW1) showed the highest disinfectant residual as well as the lowest bacterial viability. It is also shown that when the water enters the system and reaches the service reservoirs (SRs) the chlorine residues decrease and the viability increases. As distribution systems 2 and 3 apply chloramine as disinfectant the percentages of intact cells were plotted with the corresponding total chlorine residuals. In general, systems 2 and 3 show both lower viabilities throughout its networks than system 1, whereas system 2 seems to be more stable than system 3. The majority of samples in both systems showed viabilities of cells of less than 15%. On occasion in system 2 a higher percentage of intact cells of up to 30% was found when the total chlorine residual dropped below 0.8 mg/L. The same could be observed in system 3 where viabilities could reach more than 50% when the total chlorine dropped below 0.8 mg/L. The chlorine residuals in all 3 distribution systems were highest in samples taken directly after the treatment process and the viability of cells was lowest in these samples taken in the drinking water plants.

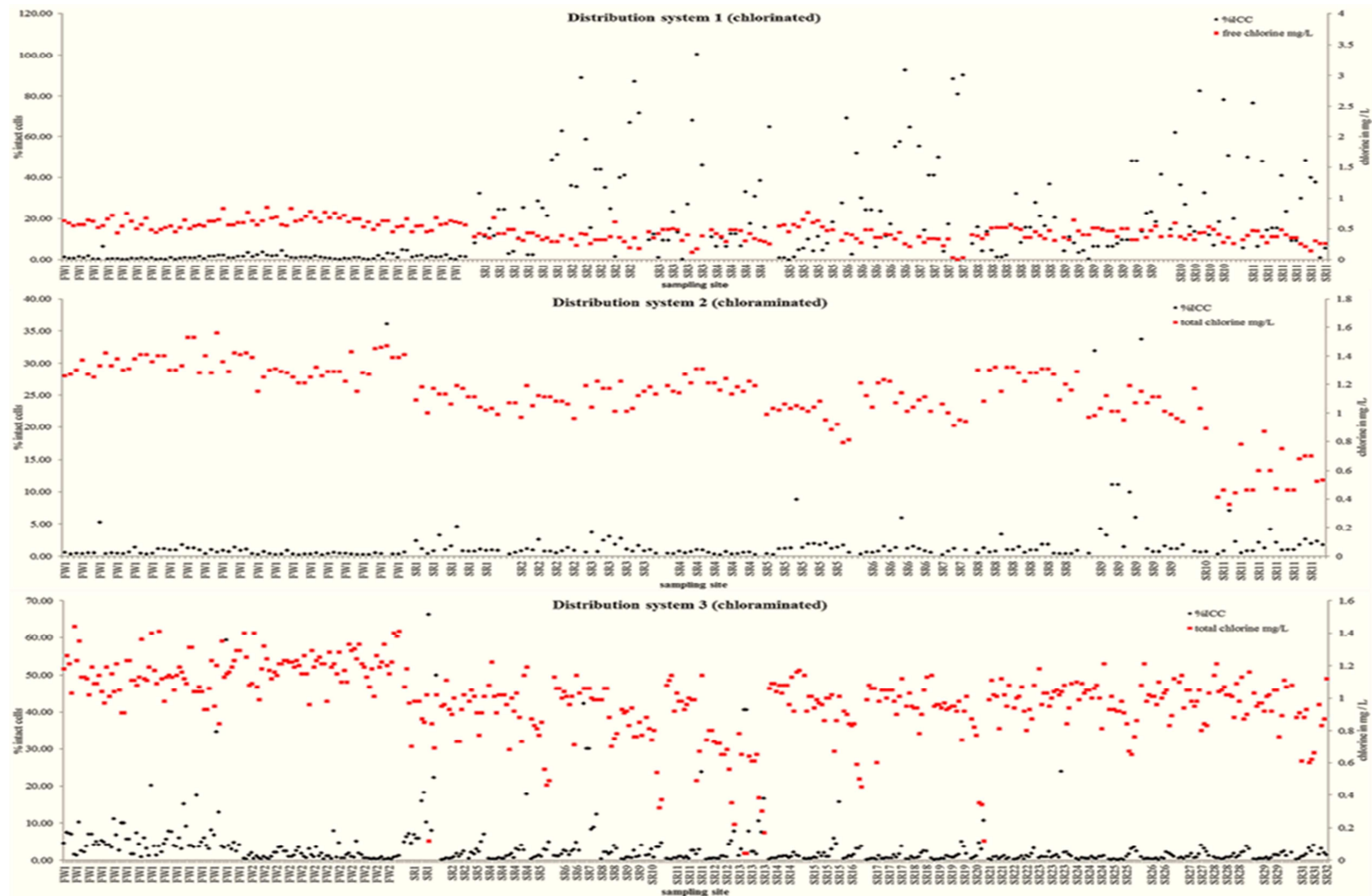


Figure 1: Percentages of intact cells and free chlorine residual (system 1) and total chlorine residual (systems 2 and 3) in 3 different distribution systems in the Scottish water network. Samples were taken from different service reservoirs (SR) in the systems as well as from drinking water treatment plants (FW).

Interestingly, in comparison with the chloraminated systems, the system disinfected with chlorine showed much higher viabilities of cells, the majority of which between 20 and 80%.

***Correlation between standard plate counts and flow cytometric analysis of bacteriological failures***

Water samples from within the Scottish Water distribution system were routinely tested for HPCs and faecal indicator bacteria using traditional plating methods. Until results were determined, the samples were stored at 4°C in the cold store. Samples that showed growth of one or more cfu of coliforms or *E. coli* per 100 mL sample volume were considered a bacteriological failure. Figure 2 (A) shows results obtained with traditional plate counts. Counts were found to vary considerably between 1 and 50 coliforms and 1 and 24 incidences for *E. coli*. HPC counts tended to vary even more between 0 and 192 counts when plates were incubated at 22°C and between 0 and 74 incidences when incubated at 37°C. No apparent correlation between HPC counts and *E. coli* and coliform counts were observed. In figure 2 (B) the free chlorine residuals of the failed water samples are shown together with percentages of intact cells. In cases where the free chlorine residual was higher than 0.5 mg/L the majority of percentages of intact cells tended to be below 15%, whereas a dramatic increase in viability could be observed as soon as the free chlorine residual dropped below 0.5 mg/L. In general, the majority of bacteriologically failed samples showed free chlorine residuals lower than 0.5 mg/L and viabilities of higher than 20%.

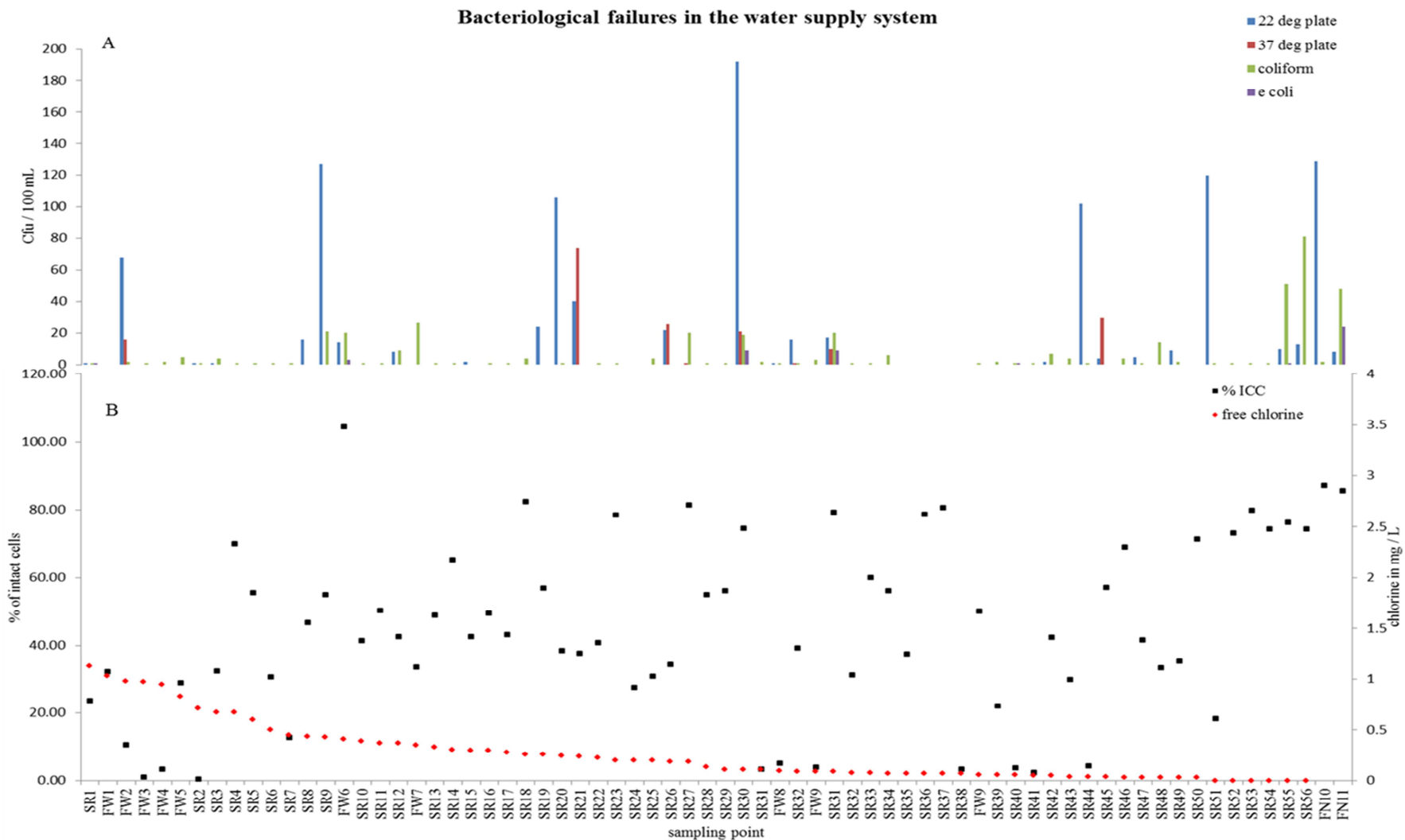


Figure 2: plate counts per 100 mL sample volume of bacteriological failures (A) and corresponding flow cytometry data (B) Samples ordered by decreasing free chlorine values. Samples from the Scottish water distribution network were chosen based on plate counts. Sampling sites were anonymised.

### ***Kinetic study of 3 distribution systems***

Over a period of 7 months samples were regularly taken from a variety of service reservoirs (SR) and water treatment plants (FW) in 3 selected distribution systems within the Scottish Water network. Flow cytometric analysis determined the total and intact cell concentrations of these samples. In figure 3 the average total and intact cell numbers are shown for each sampling point and every month. Total cell numbers in distribution system 1 showed considerable variation between  $5 \times 10^3$  and  $1.23 \times 10^5$  cells per mL throughout the examined time period. The samples taken from the treatment plant (FW1) consistently expressed the highest total cell numbers, whereas the numbers from samples taken from the different service reservoirs (SRs) displayed fewer total cells. Distribution system 2 showed less variation of total cells and tended to be more stable than system 1. In comparison to distribution system 1, system 2 showed significantly higher total cell counts between  $5 \times 10^5$  and  $4.5 \times 10^6$  cells per mL. Again the samples taken from the treatment plant consistently showed the highest bacterial numbers. Throughout the 7 month investigation period there was only little variation in cell numbers in all examined SRs and FWs and cell concentrations tended to be stable. Distribution system 3 is supplied with drinking water by 2 treatment plants. Interestingly, the lowest cell numbers found over the 7 month consistently were found in water which was sampled from the second treatment work (FW2) whereas the highest numbers could be found in samples from the first treatment plant (FW1), with cell numbers varying between  $1.5 \times 10^3$  and  $4.5 \times 10^5$  cells per mL. TCCs determined from the different service reservoirs tended to be stable on the same level through the 7 month period.

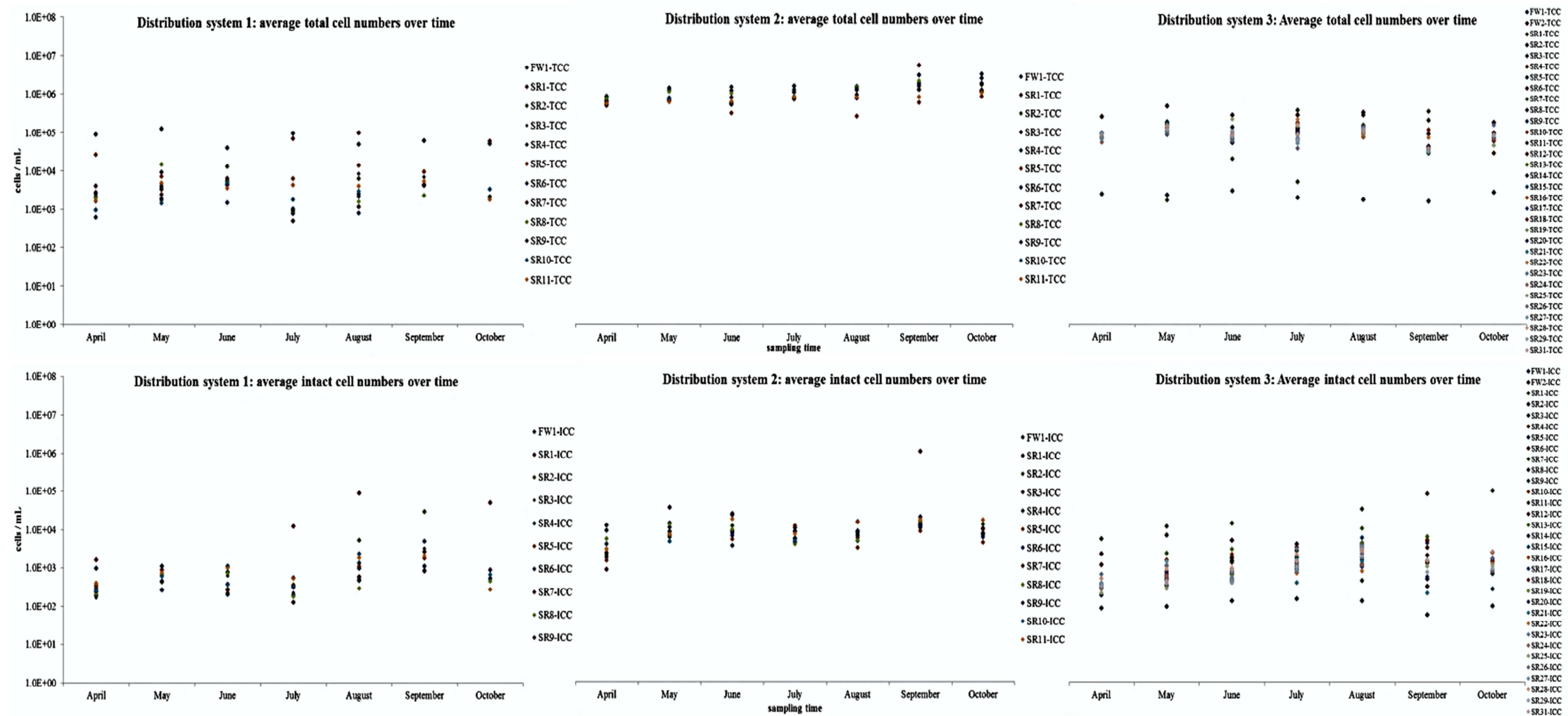


Figure 3: total cell concentrations (TCC) and intact cell concentrations (ICC) in 3 different distribution systems. Samples were taken from a variety of service reservoirs (SR) within the systems, as well as from drinking water treatment plants.



Initially, the intact cell concentrations in distribution system 1 showed numbers between  $1.5 \times 10^2$  and  $1.5 \times 10^3$  cells per mL. Over the first 3 months only slight variations were recorded and numbers tended to be stable. A rise in intact cell numbers could be determined beginning from July 2013 and the concentrations of intact cells found in the service reservoirs showed more variation. Only slight increases in intact cell concentrations were recorded with the exception of SR7, which from July until October (no data recorded in September) showed considerably higher intact cell concentrations than the average in the system. Initial intact cell concentrations in distribution system 2 were higher compared to system 1 ranging between  $1 \times 10^3$  and  $1.2 \times 10^4$  cells per mL. No significant decreases or increases in intact cell concentrations were found over the 7 months and overall intact cell numbers were stable in distribution system 2, with the exception of SR7, which showed a concentration of  $9.5 \times 10^5$  cells per mL in September 2013. The initially high variation in cell numbers decreases over the 7 month period and ranged between  $5 \times 10^3$  and  $1.4 \times 10^4$  cells per mL in October. Distribution system 3 showed the highest variation in intact cell numbers throughout the 7 month period. The lowest overall numbers were found in samples taken from the second treatment plant (FW2) ranging from  $8 \times 10^1$  to  $1.4 \times 10^2$  cells per mL. SR1 showed the highest recorded intact cell concentrations in this system between  $5 \times 10^3$  and  $8.8 \times 10^4$  cells per mL. Again, over the 7 month period the intact cell concentration tended to be stable with only few changes in numbers, but the high variation in numbers between the different sampling points was maintained throughout.

### ***Determination of the regrowth potential of 5 treated waters***

5 finally treated waters from different drinking water treatment plants in the Scottish Water distribution network were examined for their regrowth potential over a 10 day period. In addition chemical analysis assessed the nutrient concentration of these water samples. The TCC and ICC were assessed by flow cytometric analysis. Initial total cell numbers varied ranged between  $9.4 \times 10^2$  cells per mL in FW5 and  $2.4 \times 10^6$  cells per mL in FW4. Whereas FW4 is the only sample that showed a decrease in total cell concentration from  $2.4 \times 10^6$  cell per mL to  $1.4 \times 10^6$  cells per mL over the 10 day time period, the other 4 samples showed an increase in total cell numbers, with samples showing the lowest initial numbers showing the highest increase. Interestingly the variation between the cell numbers of the samples decreases over time and the samples show more similar cell concentrations between  $6.7 \times 10^5$  and  $1.7 \times 10^6$  cell per mL after the 10 days, with the exception of FW3 which showed considerably higher numbers with  $8 \times 10^6$  cells per mL. Initial intact cell concentration also showed huge differences with cell numbers ranging from  $1 \times 10^1$  cells per mL in FW3 to  $7.4 \times 10^3$  cells per mL in FW4. All samples showed significant increases in cell numbers after 3-4 days of incubation. A stagnation of the increase in intact cell numbers was found after 7 days of incubation and cell numbers ranged, with only minor alteration from day 7 to day 10, between  $6 \times 10^5$  and  $1.5 \times 10^6$  cells per mL. The only exception again was found in sample FW3, which showed no stagnation in bacterial multiplication after 7 days.

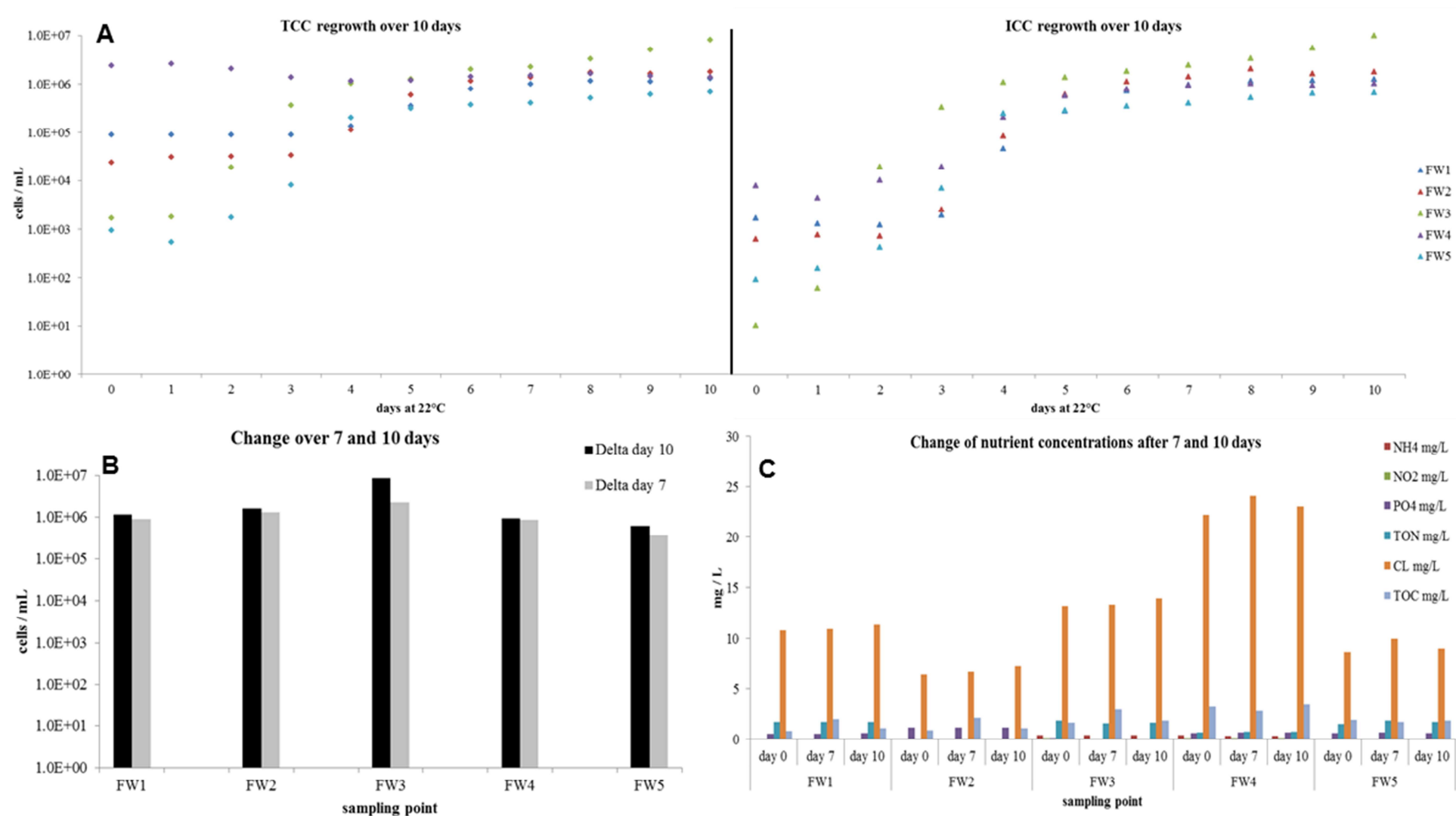


Figure 4: growth of bacterial populations of dechlorinated water samples from 5 different water sources from the Scottish Water distribution system over 10 days at 22°C. Changes in ICC and TCC (A) were recorded on daily basis. (B) Corresponding fold-increases after 7 and 10 days and (D) concentrations of total organic carbon, total organic nitrogen and phosphate at days 0, 7 and 10.

Whereas FW4 is the only sample that showed a decrease in total cell concentration from  $2.4 \times 10^6$  cell per mL to  $1.4 \times 10^6$  cells per mL over the 10 day time period, the other 4 samples showed an increase in total cell numbers, with samples showing the lowest initial numbers showing the highest increase. Interestingly the variation between the cell numbers of the samples decreases over time and the samples show more similar cell concentrations between  $6.7 \times 10^5$  and  $1.7 \times 10^6$  cell per mL after the 10 days, with the exception of FW3 which showed considerably higher numbers with  $8 \times 10^6$  cells per mL. Initial intact cell concentration also showed huge differences with cell numbers ranging from  $1 \times 10^1$  cells per mL in FW3 to  $7.4 \times 10^3$  cells per mL in FW4. All samples showed significant increases in cell numbers after 3-4 days of incubation. A stagnation of the increase in intact cell numbers was found after 7 days of incubation and cell numbers ranged, with only minor alteration from day 7 to day 10, between  $6 \times 10^5$  and  $1.5 \times 10^6$  cells per mL. The only exception again was found in sample FW3, which showed no stagnation in bacterial multiplication after 7 days. A final concentration of intact cells in sample FW3 was determined to be  $9 \times 10^6$  cells per mL which was significantly higher than in the other samples. The chemical analysis of the nutrient levels in the water samples was performed on day 0 for initial levels and on days 7 and 10 to monitor changes. For none of the sampling sites any  $\text{NO}_2$  levels were recorded. Huge variations in the Cl content were found between the different waters with FW4 showing the highest concentrations of up to 25 mg per L. The TON levels were on the same level in all samples with only minor variation except for FW2 in which interestingly no TON was recorded at all. The TOC levels within the samples varied between 0.76 and 3.4 mg per L. FWs 1-3 showed increasing values of TOC only on day 7 whereas in FW 4-5 the TOC levels maintained its initial level. The pH values of the water samples all were stable between 7.75 and 8.64.

Interestingly, only FW 3 and 4 contained any  $\text{NH}_4$  in very low concentrations of 0.38 and 0.34 mg per L which decreased over time to 0.35 and 0.31 mg per L.

#### ***Determination of regrowth potential of 42 untreated waters***

A number of different raw water samples were selected according to their plate counts and were examined for their regrowth potential by determining their TCC and ICC. Samples were analysed using flow cytometry to determine the initial cell concentrations and then stored for 7 days at 22°C in an incubator. After incubation the cell concentrations were assessed again. Regrowth potentials then were determined by examining the changes in TCC and ICC after the 7 day incubation period. Huge variations were found in coliform and *E. coli* count in the 42 different raw water samples as shown in figure 5. Coliform counts ranged between 0 in RW1 and RW2 and  $7 \times 10^4$  in RW42 per 100 mL and counts determining the presence of *E. coli* ranged from 0 in RW1 to  $1.7 \times 10^2$  per 100 mL in RW 35. No apparent correlation between counts of *E. coli* and coliform bacteria could be established although *E. coli* incidences tended to be more common in samples showing higher coliform counts. The flow cytometric analysis of the regrowth potential revealed huge variations in the different raw water samples. Some samples, especially those with lower plate counts showed an increase of total and intact cell concentrations, whereas others, mainly those with medium and high plate counts showed a decrease in cell numbers. Some of the samples showed massive decreases ( $6 \times 10^6$  cells per mL in RW 19) or increases ( $4 \times 10^6$  cells per mL in RW 10) whereas other raw waters showed only minor alteration in their bacterial populations (RW 6 and RW 29). Overall there is no apparent correlation between plate counts and flow cytometric data.

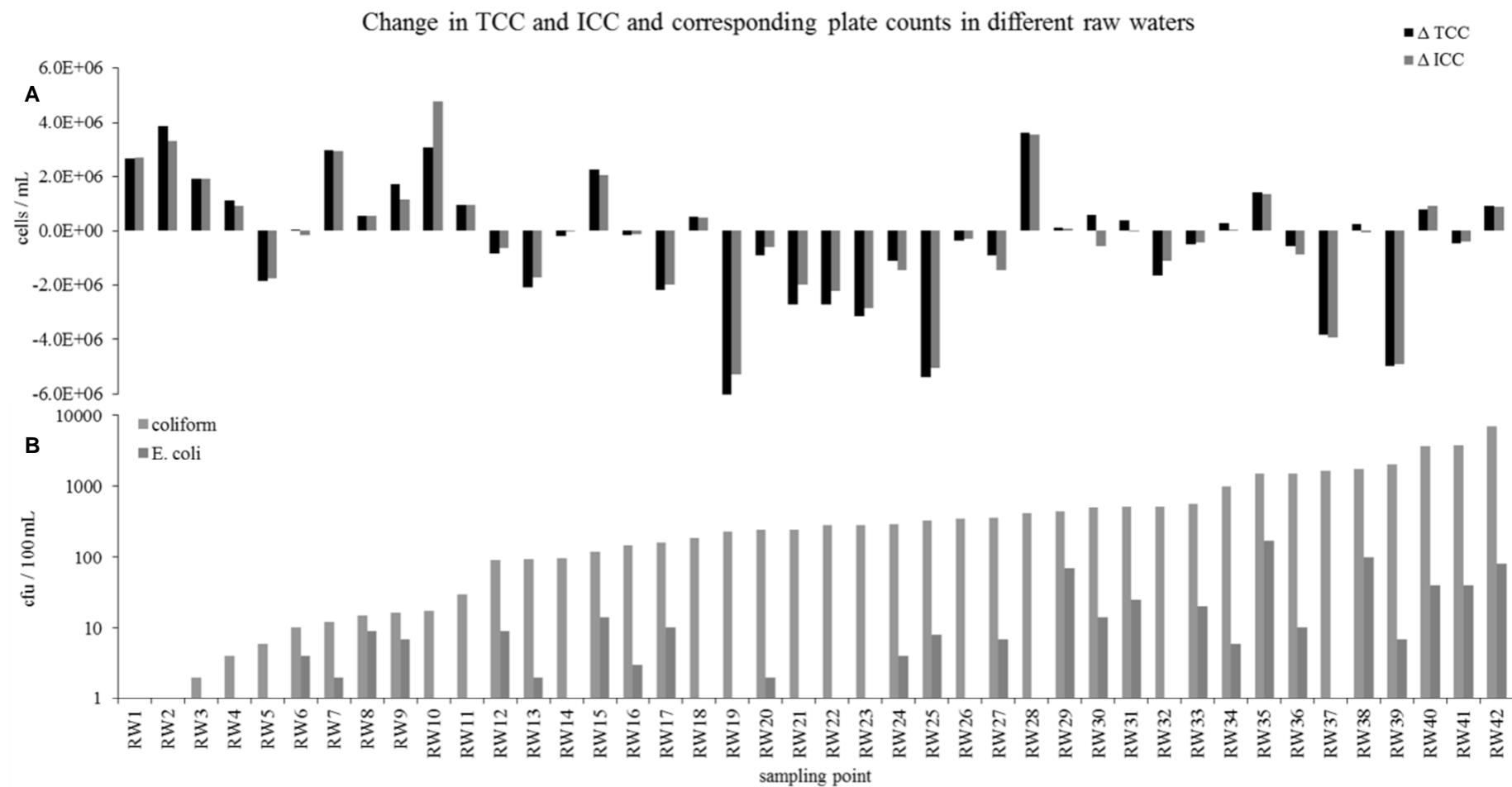


Figure 5: (A) Regrowth potentials of different raw waters expressed as in- and decreasing TCC and ICC and (B) corresponding plate counts. Samples arranged by increasing coliform counts. Samples were chosen based on plate counts. Sampling sites were anonymised.

## 2.5 Discussion

Over a period of 7 months the microbiological water quality was assessed, using flow cytometry as a rapid tool to determine the bacterial population of a sample irrespective of culturability, in 3 different distribution systems in the Scottish Water network. Two of these systems utilized chloramine as a disinfectant and in one system, chlorine was used for disinfection. It was found that when the free chlorine residual dropped below 0.5 mg per L the viabilities of bacterial cells were between 20 and 80%, occasionally reaching 100%. In the chloraminated systems elevated viabilities were found when the total chlorine in samples dropped below 0.8 mg per L. Comparing flow cytometric data to traditional plate counts of bacteriological failures, it was found that the majorities of failures showed viabilities of bacteria in excess of 20% and free chlorine residuals below 0.5 mg per L. Although no direct correlation between the number of colonies using plate counts found in the failed samples and flow cytometric analysis could be established, it can be hypothesized that increased viabilities of bacteria in the distribution system is influenced by the depletion of the disinfectant which then makes waters prone to experience bacteriological failures. The depletion of the disinfectant in a distribution system has earlier been shown to result in an increase of bacteria by Servais et al. (2004).

Substantial differences in the stability of waters could be found by comparing the 3 different networks. System 2, over the 7 months, tended to be the most stable in total and intact cell concentrations, although showing consistently the highest cell numbers with the lowest percentage of intact cells. Systems 1 and 3 both showed more variation in cell numbers in between the sampling points and both experienced higher cell viabilities. Historic data from Scottish Water shows that distribution systems 1 and 3 both experienced significant numbers of bacteriological failures in recent

months and years, whereas system 2 is considered a very stable network that only very occasionally experiences such failures. This supports the theory that high viabilities in distribution systems make water prone to experience bacteriological failures. High numbers of intact cells within a distribution system is influenced by depletion of the disinfectant which then in turn enables bacterial multiplication. Regrowth in distribution network is promoted by the availability of energy sources in the system such as nutrients and carbon sources. These growth promoting factors can be found in the treated water as nutrients not eliminated by the treatment, chemicals or coagulants used in the treatment process or piping materials used for the construction of the distribution network (LeChevallier 2003, Bucheli-Witschel et al. 2012, van der Kooij and Hijnen 1985). The regrowth potentials of 5 treated water leaving different drinking water treatment plants from the Scottish Water network were assessed. No conclusive change in nutrient levels could be established. All waters showed significant regrowth of bacteria over the 10 day incubation period and a considerable change in the viability of the cells was found from initial low percentages of intact cells to viabilities of more than 90% after incubation and depletion of the disinfectant. This showed that once the chlorine levels were depleted the bacteria present in the samples were not anymore inhibited and started utilising the provided nutrients to multiply. It has further been shown that the regrowth potential of the waters does not directly depend on the source waters by comparing the regrowth potentials of treated waters FWs to regrowth potentials of raw waters RWs. The raw waters did not experience such significant increases in cell numbers or viabilities. Being not depleted of most of the natural bacterial population more cells were competing for nutrients and therefore the raw waters tended to be more stable.



In conclusion, the presented data supports previous findings that bacterial concentrations in drinking water depend on the disinfectant residual. Our findings suggest that a target disinfection residual of 0.5 mg/L free chlorine in chlorinated systems and 0.8 mg/L in chloraminated systems has to be maintained to prevent elevated intact cell concentrations. The study further underlines that regrowth potentials of waters do not depend on the water source but rather on the efficacy of the treatment process.

## **2.6 Acknowledgements**

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## **Conclusion**

The two studies undertaken during this project aimed to contribute to the acceptance of FCM as an operational tool for water quality assessment. Whereas the first study focussed on the customer's perspective, the second study had its focus on the water suppliers perspective and aimed to promote FCM as a decision making tool. The data collected from the experiments suggest that a target disinfection residual of 0.5 mg/L free chlorine in chlorinated systems and 0.8 mg/L in chloraminated systems has to be maintained to prevent elevated intact cell concentrations. Although elevated intact cell concentrations do not automatically suggest a risk to human health, they are an indication for an insufficient disinfection process. In those distribution systems then the presence of pathogens combined with insufficient disinfection and unsuppressed regrowth may lead to health risk upon consumption. The study further underlines that regrowth potentials of waters do not depend on the water source but rather on the efficacy of the treatment process. Another important factor contributing to the intact cell numbers is stagnation of the water. Especially in the piping material in customers houses, where the chlorine level tends to be the lowest, water stagnation was found to have a dramatic effect. Although further studies are necessary, the collected data indicates that the proportions of intact cells and their correlation with disinfectant levels together with the regrowth potential may be more reliable than the currently used standard tools and when gaining more acceptance might replace older methods such as HPCs.

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